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Brucella-positive raw milk cheese sold on the inner European market: A public health threat due to illegal import?

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ABSTRACT

Travel and migration are the major drivers of human brucellosis in Western Europe. The infection is usually transmitted through the consumption of unpasteurized milk or dairy products in or from endemic regions. Although eradicated from livestock in Germany and most Member States of the European Union, considerable numbers of domestic human brucellosis cases have been reported annually. The actual source of these autochthonous cases in non-endemic countries remains to be elucidated.

We therefore evaluated the presence of *Brucella* spp. in 200 cheese samples originating from endemic countries which were sold at weekly markets, in supermarkets and by delis in Berlin (Germany) as well as online. The cheese samples included loose, non-labelled and pre-packed, labelled cheese of five types (brine, cream, soft, semi-hard and hard cheese), made from bovine, ovine and caprine milk. The cheese was mainly declared as raw milk cheese by the retailers. We screened for and confirmed the presence of *Brucella*-DNA in cheese using genus-specific quantitative real-time PCRs targeting IS711 and *bcsp31*, respectively. The molecular prevalence of *Brucella* was 20.5% (n = 41), but viable Brucellae could not be isolated from the positively tested samples using classical culture methods. The logistic regression model indicated that *Brucella* was significantly more often detected in late summer purchases ($p = 0.036$) as well as in cheese from Bulgaria, France, Greece and Turkey ($p = 0.017$). In contrast to the vendor information, essentially only three positive cheese samples were made from raw milk. Moreover, positive samples clustered at certain vendors which indicates large-scale illegal imports.

In summary, *Brucella* in imported raw milk cheese seems to be still a challenge for food safety standards in the European Union. Uncontrolled import of dairy products from endemic regions might explain human *Brucella* infections acquired in non-endemic EU countries.

1. Introduction

1.1. Epidemiology of animal brucellosis in Europe

Brucellosis is one of the most common zoonoses throughout the world. The causative agents are Gram-negative bacteria of the genus *Brucella,* comprising twelve species. The main burden of human disease is due to *B. melitensis* and *B. abortus*, transmitted from sheep and goats and from cattle and other Bovidae, respectively. In the European Union (EU) brucellosis has been successfully eradicated from livestock in most Member States (MS) including Germany leading to the status officially free of bovine brucellosis caused by *B. abortus* (Officially Brucellosis Free, OBF) as well as officially free of ovine and caprine brucellosis caused by *B. melitensis* (Officially *B. melitensis* Free, ObmF) (EC, 2003). A MS or a region of a MS may be declared OBF if neither a case of abortion due to *Brucella* infection nor isolation of *B. abortus* has been recorded for at least three years and at least 99.8% of the herds monitored with serological methods have achieved OBF status each year for five consecutive years (EEC, 1964). By the end of 2016, Bulgaria, Croatia, Cyprus, Greece, Hungary, Italy, Portugal, Spain and the United Kingdom (Channel Islands Jersey and Guernsey) were not yet OBF (EFSA and ECDC, 2017). A MS or a region of a MS may be declared ObmF if at least 99.8% of the ovine and caprine holdings are ObmF, or if the following criteria are met: (i) ovine or caprine brucellosis is a disease that has been compulsorily notifiable for at least five years; (ii) no case of ovine or caprine brucellosis has been officially confirmed for

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at least five years; and (iii) vaccination has been prohibited for at least three years (EEC, 1991). The eight MS that by the end of 2016 had not yet gained a country-level ObmF status were Bulgaria, Croatia, Greece, Italy, Malta, Portugal and Spain (EFSA and ECDC, 2017).

1.2. Epidemiology of human brucellosis in Europe

Brucella spp. can be directly transmitted from infected animals and contaminated tissues to humans via inhalation or through skin lesions which is an occupational risk for veterinarians, abattoir workers and farmers, particularly in endemic regions. However, the ingestion of contaminated raw milk and dairy products poses the major public health risk. In milk and products thereof, *Brucella* is controlled most effectively by pasteurization or sterilization before marketing or by further processing into dairy products. *Brucella* spp. have a D-value of 6–12 s at 65.6 °C and a Z-value between 4.4 and 5.6 °C in milk (Rowe, 2014). However, the number of Brucellae in raw milk varies widely between infected animals depending on the species, the physiological status of the animal and the route of infection. Virulent *Brucella* spp. are excreted in milk by 12–44% of infected cows and up to 60% of infected goats. Few animals have been reported to shed up to 10^4 cfu *B. abortus* per ml raw milk (so-called super shedders) though the majority of infected animals shed less than 10^3 cfu/ml (Capparelli et al., 2009).

In Western Europe, brucellosis became a rare zoonotic disease in the last decades owing to a strict eradication program. Nonetheless, it is a severe disease with most of the brucellosis cases reported in the EU hospitalized (EFSA and ECDC, 2017). The acute manifestation usually presents with flu-like symptoms that may persist and progress if untreated to a chronically incapacitating disease with severe focal complications such as spondylitis, neurobrucellosis or *Brucella* endocarditis (Adone et al., 2013). From 2010 to 2015, the number of human brucellosis cases reported in EU MS remained stable. However, an increase of 35.2% was recorded in 2016, mainly due to an increased number of notifications in Italy, where case numbers more than doubled compared with 2015. As in previous years, the highest notification rates of brucellosis were reported by the three EU MS that were not OBF/ObmF (namely Greece, Italy and Portugal), together accounting for 73.6% of the cases in 2016. The proportion of infections acquired domestically in the EU decreased from 79.7% (401 cases) to 37.6% (194 cases) between 2012 and 2016 (EFSA and ECDC, 2017). A similar pattern was seen in non-endemic Germany. The number of reported human brucellosis cases in Germany was constant in 2012, 2013 and 2014 (with 28 cases per year), but interestingly almost half of the cases in 2014 (46%) were autochthonous. The remaining ones could be traced back to endemic countries, most frequently to Turkey (n = 6, 21%) (RKI, 2014). Indeed, brucellosis has evolved from an endemic occupational disease among the German population in the 1960s to a travel-associated foodborne zoonosis, primarily affecting immigrants nowadays (Al Dahouk et al., 2007b). In Germany, the risk to acquire brucellosis has proven to be 30 times higher in people with a Turkish immigration background than in people of German origin. Nevertheless, 26% of the brucellosis cases reported in Germany were assumed to be domestically acquired (Al Dahouk et al., 2007b). Illegally imported products of animal origin (POAO) are a well-known source for the transmission of zoonotic pathogens despite existing prohibitions and strict controls (Jansen, Grabowski, & Klein, 2015) and considerable amounts of POAO are seized and destroyed every year in Germany, accumulating to more than 20,000 kg annually at the biggest German international airport (Jansen et al., 2016). Many of these products may harbour zoonotic pathogens, including *Brucella* spp. (Beutlich et al., 2015), and are known to pose a public health threat, but these previous findings cannot explain the extent of autochthonous human brucellosis cases in non-endemic Germany.

Therefore, we assumed that raw milk cheese originating from endemic countries and sold at weekly markets, in supermarkets and deli retail shops in Berlin as well as from online shops are a potential domestic source of *Brucella* in a non-endemic country.

1.3. Diagnostic methods for the detection of Brucella spp. in food

As no methodological guidelines have been published by the International Organization for Standardization (ISO), the World Organization of Animal Health (OIE) Manual regulates the gold standard for the diagnosis of brucellosis. Laboratory diagnosis of *Brucella* comprises direct detection methods using molecular and culture based techniques as well as indirect serological tests which are mainly applied to milk and blood.

As bacteria are usually widely dispersed throughout the contaminated foodstuff, molecular methods, such as quantitative real-time polymerase chain reaction (qPCR), are most suitable for the sensitive, reliable and quick detection of slow-growing *Brucella* spp. in food (Al Dahouk, Nöckler, & Tomaso, 2009). The best validated methods are based on the detection of *Brucella* genus-specific sequences, such as the 16S/23S rRNA genes, the *bcsp*31 gene encoding a 31-kDa cell surface membrane protein or the IS711 insertion sequence (Baddour et al., 2008; Ouahrani et al., 1996). The target sequence IS711 is often used for screening to increase the analytical sensitivity of molecular detection systems because this non-coding intergenic DNA sequence occurs in several copies within the *Brucella* genome (Tomaso et al., 2010). Initially, genus-specific PCRs were developed to rapidly identify bacterial isolates but have been used ever since to detect *Brucella* DNA directly in clinical samples (Al Dahouk et al., 2007a; Dauphin, Hutchins, Bost, & Bowen, 2009). The specificity of genus-specific *Brucella* PCRs targeting *bcsp*31 and IS711 has proven to be close to 100% in dairy matrices irrespective of the natural microbiome (Marianelli et al., 2008). DNA extraction methods are known to influence the analytical sensitivity of PCR assays (Tomaso et al., 2010). In artificially contaminated cheese the detection limit of cultural methods including enrichment was approx. 30 colony forming units (cfu)/ml, whereas < 10 cfu/ml could be detected with a conventional genus-specific semi-nested PCR (Tantillo, Di Pinto, & Buonavoglia, 2003). In cheese made from milk of naturally infected sheep and goats, a conventional *bcsp*31 PCR tested 46% of the samples *Brucella*-positive but bacterial isolation was not successful at all (Tantillo, Di Pinto, Vergara, & Buonavoglia, 2001). Hence, the number of pathogens in the cheese was either too small or the pathogens were inactivated during the ripening process or thermal treatment of the primarily contaminated milk. Additionally, numerous bacteria in the cheese microbiome are important for the production and compete with the growth of pathogens such as *Brucella*.

2. Material and methods

2.1. Cheese samples

A total of 185 cheeses were purchased in Berlin (Germany) at (i) weekly ethnic markets with Turkish traders and multicultural customers predominating [Maybachufer (n = 98) and Winterfeldtplatz (n = 27)], (ii) in ethnic delis (n = 14) and supermarkets located in districts with a high number of immigrants [Neukölln (n = 26) and Schöneberg (n = 20)]. Thereof, 146 (79%) were prepacked, sealed and labelled, whereas 39 (21%) were loose, non-prepacked and non-labelled. A total of 15 prepacked cheese samples were purchased (iii) online via the trading platform Ebay® to obtain a sample size of n = 200. The purchases were done (i) in early summer (May (n = 61) and June (n = 15)) and (ii) in late summer (August (n = 56) and September (n = 68)) of the year 2011.

The cheese samples were made predominantly out of sheep's milk (n = 96; 48%), followed by goat's milk (n = 45; 22.5%) and cow's milk (n = 13; 6.5%), whereas 46 (23%) cheeses were made out of a mixture of these three different types of milk. The majority of the samples were sold as raw milk cheeses (n = 156; 78%) according to the label or the information provided by the vendor. To allow for comparison, 44

cheeses (22%) made out of pasteurized milk were included in the study. We mainly investigated short ripened cheeses such as feta and brine cheese (n = 89), soft cheese (n = 36) and cream cheese (n = 30) as well as short ripened semi-hard cheese (n = 17 Tulum, n = 5 Kaschkaval, n = 3 Halloumi). Additionally, we evaluated 20 hard cheeses (e.g. Pecorino, Manchego). Corresponding to the label with the official EU identification mark in accordance with Regulation (EU) 853/2004 and/or the vendor information, the countries of origin were Turkey (n = 50), France (n = 47), Bulgaria (n = 42), Greece (n = 34), Spain (n = 5), Italy (n = 4), Belgium (n = 2), Croatia (n = 2), Cyprus (n = 3), Lebanon (n = 2) as well as the Czech Republic, Germany and The Netherlands with n = 1 each. For 6 loose cheese samples the country of origin could not be determined.

2.2. Bacterial culture

A total of 10 g from two different strata of each cheese sample (outer rind and inner core) were homogenized with a BagMixer® Stomacher (Interscience, Paris, France) in 90 ml Farrell's selective medium in accordance with the OIE detection standard (OIE, 2016). This liquid culture was incubated at 37 °C with and without 5% CO_2 over 6 weeks and sub-cultured weekly on solid *Brucella* Agar and Farrell's selective agar (OIE, 2016). Last but not least, 16S rRNA sequencing was carried out for grown colonies to identify phylogenetic affiliation of all bacterial isolates.

2.3. DNA extraction and quantitative real-time PCR methods

From the individual cheeses, 5 g each of the outer rind and of the inner core were homogenized separately in 25 ml phosphate buffered saline (PBS) using 1 g of silica sand and ten ceramic spheres in a FastPrep-24™ Homogenizer (MP Biomedicals, Eschwege, Germany). The cheese samples were centrifuged and, subsequently, we extracted and purified DNA from the supernatant using the commercial DNeasy mericon® Food Kit (Qiagen, Hilden, Germany). All cheese samples were tested with the IS711 real-time PCR and the *bcsp31* real-time PCR (Al Dahouk et al., 2007a). For IS711 real-time PCR assays, we used the primer and probe sequences described by Matero et al. (2011). To reveal possible inhibitory effects, real-time PCRs were performed with the Qiagen Pathogen Detection Kit®, including a synthetic internal amplification control (Qiagen).

Pretesting was carried out with special focus on *B. melitensis* due to the major epidemiological impact of this species on human brucellosis in Germany. The sensitivity of the IS711 real-time PCR was assessed with 88 *B. melitensis* strains representing all biovars of the species (bv 1–3), including 85 human and animal field isolates (18x bv 1, 36x bv 2, and 31x bv 3) and 3 *B. melitensis* reference strains (16M, 63/9, Ether). For specificity testing, we used (i) raw milk sampled from 15 sheep and 6 goats of the experimental farm of the German Federal Institute for Risk Assessment and (ii) a representative panel of 38 commercial starter cultures for cheese production (IP Ingredients GmbH, Süderlügum, Germany). The detection limit (analytical sensitivity) of the IS711 real-time PCR was determined in comparison to the established *bcsp31* real-time PCR in spiked cream, soft and hard goat cheese samples (serial decimal dilution series of *B. melitensis* 16M DNA between 2 ng and 2 fg/g) in due consideration of the theoretical genome weight of 3.38 fg for *B. melitensis* 16M (Al Dahouk et al., 2007a). A cheese was assumed to be *Brucella*-positive if both genus-specific qPCRs independently showed positive results. Gel analysis of the PCR products was performed for all cheese samples tested *Brucella* positive in the *bcsp31* real-time PCR. The expected amplicon size of 223 bp helped to exclude unspecific primer annealing.

Though nowadays more detailed analyses on species and strain level are possible, the regulation (EU) 853/2004 does not consider the relevance of subtyping but may impose restrictions if members of the genus *Brucella* are isolated from food. Similarly, international veterinary regulations also impose restrictions on animal movements and trade no matter which *Brucella* species is detected (OIE, 2016). In terms of legal evaluation, species analysis therefore did not provide any added value in our study.

2.4. Enzymatic analysis to confirm milk heat treatment

To determine the raw milk content of the cheeses, a subset of molecular positive samples was analyzed for alkaline phosphatase activity in line with DIN 10337:1993–12 (DIN, 1993) using a commercial assay (Hardy Diagnostics, Santa Maria, CA, United States of America). Inhibitors such as the cheese (mold) rind and condiments or spices were removed, cheese samples were homogenized in PBS, and positive and negative controls were run with each test. Testing alkaline phosphatase activity is the national standard recommended for the confirmation of successful pasteurization in the "Official Collection of Methods of Analysis and Sampling" in accordance with §64 of the German Food and Feed Act.

2.5. Statistics

Data were analyzed with descriptive statistics and processed in Microsoft® Excel 2011. Calculations were performed in RStudio. Chi-square ($\chi 2$) tests and a logistic regression model were used to evaluate differences between the impact of various independent variables on *Brucella* detection in cheese samples, and odds ratios were calculated. Cheeses were grouped according to the milk used for production [raw milk or pasteurized milk as well as (i) cow's, (ii) sheep's, (iii) goat's and (iv) mixed milk] and according to the manufacturing style [(i) feta and brine cheese, (ii) cream cheese, (iii) soft cheese, (iv) semi-hard cheese and (iv) hard cheese]. Groups were also formed for the country of origin of the cheeses including Bulgaria (BG), Croatia (HR), Cyprus (CY), Czech Republic (CZ), France (FR), Germany (DE), Greece (GR), Italy (IT), Lebanon (LB), The Netherlands (NL), Spain (ES), Turkey (TR) and of unknown origin. Regarding the sale, we grouped the cheeses into (i) prepacked or (ii) loose, sold (i) in supermarkets and ethnic delis, (ii) by market vendors or (iii) online, and according to season of the sale [(i) early summer or (ii) late summer].

3. Results

3.1. Preliminary experiments testing sensitivity, specificity and the detection limit of genus-specific Brucella qPCRs in cheese matrices

The IS711 and *bcsp31* qPCRs were able to detect all 85 *B. melitensis* field strains and reference strains. One false-positive result (4.7%) in sheep's milk was recorded with the IS711 qPCR, but not confirmed by *bcsp31* qPCR. Seven false-positive results (18.4%) occurred in the 38 starter cultures tested, which neither were confirmed with the *bcsp31* qPCR. The IS711 qPCR was able to detect substantially lower concentrations of the pathogen in spiked cheese samples than the *bcsp31* qPCR. Using the theoretical genome weight of *B. melitensis* 16M (= 3.38 fg) for the calculation of genome equivalents (GE), the analytical detection limit of IS711 versus *bcsp31* qPCR in cream cheese was < 1 vs. 6 GE/g, in soft cheese 6 vs. 59 GE/g and in hard cheese < 1 vs. 6 GE/g. In addition, the repeatability of *Brucella* detection using the IS711 qPCR was more stable in test-retest reliability analyses of the spiked samples compared to the *bcsp31* qPCR, particularly in cream and soft cheese.

3.2. Prevalence of Brucella in cheese sold at retail level

A total of 134 and 42 cheese samples were tested positive using the IS711 and the *bcsp31* real-time qPCR, respectively. Forty-one out of 200 (20.5%) cheese samples were finally classified as *Brucella* positive since both genus-specific PCRs independently showed positive results

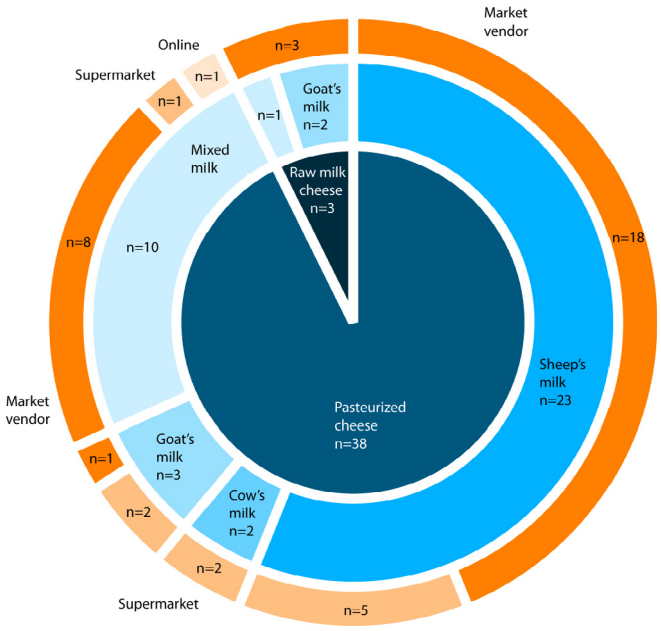

Fig. 1. Heat treatment, type of milk used for cheese production and location of purchase of the 41 *Brucella* positive cheese samples.

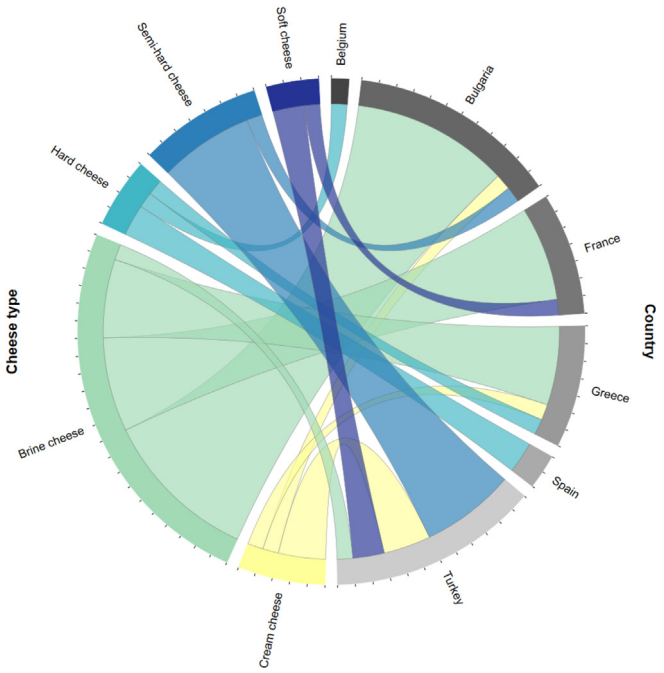

Fig. 2. Chord diagram displaying the inter-relationship between cheese type and country of origin of the *Brucella* positive cheese samples (n = 41). Arc lengths on the outer circle are proportional to total quantities.

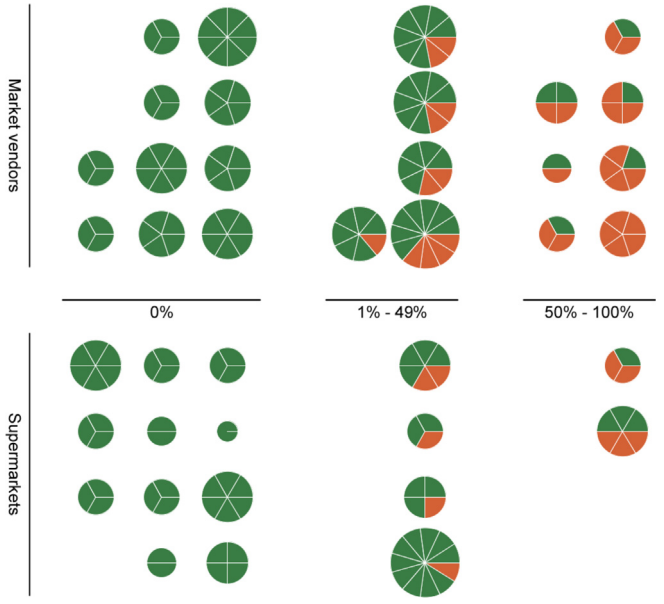

Fig. 3. Sale of *Brucella* DNA positive (red, n = 41) and negative (green, n = 159) cheese by market vendors and supermarkets. Each circle represents a retailer and is proportional to the sample size. The percentages (0%, 1–49%, 50–100%) indicate the relative abundance of *Brucella* positive compared to *Brucella* negative cheese samples found in each location.

(Fig. 1). Cheese made from pasteurized sheep's milk and sold unlabeled or loose by market vendors was the most frequent type (n = 19, 46%) associated with the presence of *Brucella* DNA. However, the logistic regression model neither detected a significant difference between cheese originating from weekly markets (p = 0.06) and those sold by delis, supermarkets and online, nor between loose and prepacked cheese (p = 0.85).

Considering the information provided by the vendor, 4 *Brucella* positive cheese samples were made from pasteurized milk, whereas 37 positive cheese samples were sold as raw milk cheese. Actually, only 3 out of the 41 positive cheese samples revealed alkaline phosphatase activity and, therefore, 38 samples had to be classified as pasteurized cheese. The prevalence of *Brucella* DNA was not significantly higher in cheese samples made from raw milk than from pasteurized milk (p = 0.08), and showed no difference among the various cheeses (p = 0.59). *Brucella* DNA was detected with the same frequency in cheese produced in EU MS (23%) and in non-EU MS (22%). The comparison of individual countries of manufacture, however, revealed a significantly higher prevalence of *Brucella* DNA in cheese originating from Bulgaria (p = 0.00730), France (p = 2.1e-05), Greece (p = 0.00146) and Turkey (p = 0.00050) (Fig. 2).

Brucella positive cheese samples were purchased significantly more often in late summer (n = 32, 78%) than in the early summer months May and June (n = 9, 22%) (p = 0.0173). The spatial distribution revealed that nine vendors sold > 50% of the *Brucella* positive cheese samples, including seven vendors at weekly markets and two supermarkets (Fig. 3).

Although amplification of *Brucella* DNA was successful, viable Brucellae could not be isolated using culture methods. The cheese microbiome was largely suppressed by Farrell's selective medium but numerous bacterial species could be still isolated comprising both environmental bacteria as well as human pathogens such as *Acinetobacter* spp., *Klebsiella* spp., *Enterococcus faecium*, *Pseudomonas aeruginosa* and *E. coli*.

4. Discussion

We evaluated the prevalence of *Brucella* in cheeses sold to final consumers by retailers on the German market using standard culture methods and genus-specific qPCRs. Our major goal was to assess whether autochthonous human brucellosis cases in Germany can be attributed to imported dairy products from endemic regions.

4.1. Limits of molecular Brucella detection and bacterial isolation

In the cheeses under study, *Brucella* was detected in 41 (20.5%) out of 200 samples by two independent genus-specific qPCR methods. The analytical sensitivity of the IS711 qPCR is known to be higher than the one of *bcsp31* qPCR which is why 134 cheese samples were tested

positive only by this assay. The single false-positive result in the *bcsp31* qPCR was probably caused by contamination with *Ochrobactrum intermedium* which is phylogenetically one of the closest neighbours of *Brucella*. At least, *O. intermedium* was identified by 16S rRNA gene sequencing from the corresponding bacterial isolate (data not shown). The molecular detection of foodborne pathogens in a complex food matrix is a technical challenge because high concentrations of endogenous *Taq* polymerase-inhibitors remain after nucleic acid extraction leading to inhibitory effects and false negative amplification (Wilson, 1997). We therefore applied the Qiagen Pathogen Detection Kit® which benefits from an internal amplification control to record potential inhibitory effects.

In our study, *Brucella* could not be isolated from the PCR positive cheese samples using classical culture methods. *Brucella* cultures are demanding and the isolation of the pathogen particularly from complex matrices such as food is only rarely successful (Serpe, Gallo, Fidanza, Scaramuzzo, & Fenizia, 1999), even by means of selective media and enrichment. In contrast, the analytical sensitivity of real-time PCR techniques for pathogen detection in food is relatively high compared to culture-based methods. However, the applied PCR setup cannot prove the presence of live bacteria; this drawback may lead to significant under- or overestimation of the results. Isolation of *Brucella* spp. from cheese strongly depends on the initial contamination level of the milk, the type of heat treatment, homogenization and fat-standardization of the milk, the ripening process and storage conditions (humidity, temperature), the pH value and salt content of the cheese as well as the period between production and testing (Davies & Casey, 1973; Kasimoğlu, 2002; Sabbaghian, 1975). In accordance with the OIE standard, we cultured both superficial strata and the core of each cheese product (OIE, 2016) but even though enrichment culture was applied and samples were carefully homogenized before cultivation of an appropriate volume to avoid over-dilution, growth of *Brucella* spp. could not be determined.

4.2. Brucella in raw milk cheese

The consumption of unpasteurized milk or other dairy products from endemic regions due to imports or travels are by far the most important transmission routes for human brucellosis reported in North America and Northern Europe (Corbel, 2006). Therefore, our purchases particularly focused on raw milk cheese from endemic countries. However, we were not able to confirm the vendors' information regarding the raw milk content. In total, 37 of the 41 positive samples were sold as raw milk cheese but only 3 of them proved to contain raw milk. Hence, 93% of the cheese samples in the positively tested subset were made out of pasteurized milk. The vendors who committed food fraud might have been either unaware of this fact or wanted to satisfy their customers. While this fraudulent practice does not put consumers at risk, consumer deception is obvious. Raw milk cheese manufacturing requires the utmost accuracy, care and, above all, an exceptional emphasis on quality. The use of raw milk is known to positively affect flavour, texture and sensory aspects of cheese. Raw milk has to comply in chemical and microbiological respects with the generally prevailing public understanding. According to the regulation (EU) 853/2004, raw milk is defined as the secretion of the mammary gland of farmed animals that has not been heated to more than 40 °C or undergone any treatment that has an equivalent effect. Microbiological criteria for the production and collection of raw milk are laid down in the regulation (EU) 853/2004 and particularly with regard to brucellosis, raw milk must be from (i) cows or buffaloes belonging to a herd which, within the meaning ofDirective 64/432/EEC, is free or officially free of brucellosis in accordance with OIE standards; (ii) sheep or goats belonging to a holding officially free of brucellosis in accordance with OIE standards or free of brucellosis within the meaning of Directive 91/68/EEC; or (iii) females of other species susceptible to brucellosis and belonging to herds regularly checked for brucellosis under a control plan that the competent authority has approved. The same requirements are implemented for imported cheeses from endemic regions (EU, 2004).

We detected *Brucella* in three raw milk cheeses from different EU MS, namely France, Greece and Spain. France has been officially free of bovine brucellosis since 2005, and no brucellosis outbreaks in sheep and goats have been reported since 2003. However, we detected *Brucella* DNA in a French raw goat milk soft cheese. The existence of *Brucella*-positive raw milk soft cheese produced in France was confirmed in the year after our study, when an autochthonous case of human brucellosis originating from cattle was diagnosed. The French investigation demonstrated that the patient was infected by *B. melitensis* bv 3 contaminated soft cheese (Reblochon) made from raw milk at a dairy farm in the French Alps. The official authorities hypothesized transmission either by a congenital case with a dam infected more than a decade ago when bovine brucellosis was still endemic in this region or by re-introduction of the pathogen from wildlife (Mailles et al., 2012). Indeed, the persistence of *Brucella* in alpine ibex and its spillover to livestock poses an ongoing risk for brucellosis in France (Mick et al., 2014). Nowadays, France is considered to be ObmF and brucellosis has not been reported in small ruminants since 2003 (Perrin et al., 2015). The ripening process of the contaminated soft cheese from France, we detected in our study, usually takes 3 weeks and is mainly based on lactic fermentation. *Brucella* spp. are able to survive weeks to months in acidic environments found in comparable dairy products (Davies & Casey, 1973; El-Daher, Na'was, & al-Qaderi, 1990; Keogh, 1971). Furthermore, the number of live bacteria decreases more slowly in cheese than in milk or yogurt (El-Daher et al., 1990). In addition to the ripening period, pH and water activity are considered as major factors influencing the survival of *Brucella* in dairy products. The optimal pH for survival and growth of *Brucella* spp. at 37 °C ranges between 6.6 and 7.4 (ICMSF, 1996). However, the bacteria can still survive under extreme alkaline (maximum pH 8.4) (Zobell & Meyer, 1932) and acidic (minimum pH 4.1–4.5) conditions (Lerche & Entel, 1959). The fat content of a dairy product may also affect bacterial survival. For instance, yogurts with different fat contents artificially contaminated with *Brucella abortus* 1119–3 (9.7 log cfu/ml) did not contain viable bacteria anymore after two days (10.0% fat), three days (1.5% fat) and five days (3.5% fat) (Falenski et al., 2011).

Despite the resistance of *Brucella* spp. towards harsh environmental conditions, raw milk may be used for cheese production if the maturation period lasts more than two months (62 days). However, authorization of the competent authority is needed if the raw milk originates from animals which are not officially free from brucellosis but have been negatively tested, or which have been vaccinated against brucellosis within an approved eradication program, and which do not show any symptoms of the disease (EC - European Commission, 2004). We detected *Brucella* DNA in two hard cheeses made from raw milk and produced in Spain (goat's milk) and Greece (mixed milk), both known for endemic regions within the country. A maturation period of at least two months for cheese made out of contaminated milk may not be sufficient for effective elimination of the pathogen: *B. melitensis* 16M was still detected after 50 days at 3 log cfu/mL in brine cheeses made from artificially contaminated goat milk (9.7 log cfu/mL) and ripened at 4 °C despite a pH of 5.0 and a water activity (a_w) of 0.90 (Mendez-Gonzalez et al., 2011). In 1965, a brucellosis outbreak in London (United Kingdom) was traced back to an Italian hard cheese (pecorino) made from unpasteurized sheep's milk, which has ripened for more than 90 days (Galbraith, Ross, de Mowbray, & Payne, 1969). Clasessens and Ring (1996) reported that *B. melitensis* could even survive up to 90 days in ripened brine cheese made from raw sheep's and goat's milk. Consequently, the 62 + day rule may not guarantee complete clearance of dangerous bacteria in unpasteurized cheese and needs to be revised thoroughly.

4.3. Brucella in pasteurized cheese

Milk from animals belonging to a herd that is not officially free from brucellosis may be used after heat treatment such as pasteurization at 72–75 °C for 15–30 s with the authorization of the competent authority (EC - European Commission, 2004). We detected *Brucella* in cheese made out of pasteurized milk and originating from four EU MS (Belgium, France, Greece and Spain).

A single Belgian hard cheese made from cow's milk contained *Brucella* DNA. Belgium was awarded OBF/ObmF status in 2003, but an outbreak of brucellosis in cattle caused by *B. suis* bv 2 was reported in 2012, probably due to the spillover from wild boars (Fretin et al., 2013). This could be the cause for a positive test result in pasteurized cheese from Belgium. In 2013, all dairy herds of the country were sampled for a serological screening in bulk milk (n = 9460) and proved to be negative (EFSA and ECDC, 2014).

In total, six French brine cheese made from sheep's milk contained *Brucella* DNA. All pasteurized French cheese samples which were tested positive in our study were made in Corsica. Actually, France is declared OBF/ObmF, including Corsica (*personal communication* Claire Ponsart, Head of EU/FAO/OIE Reference Laboratory for Brucellosis, Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail (ANSES)). In 2013, however, human brucellosis cases in the metropolitan areas of France could be traced back to Corsican raw milk cheese, which highlights the importance of this transmission route (Heuzé, Ruello, & Macarry, 2014, p. 2).

Among the cheeses imported from the non-OBF/non-ObmF MS Greece, seven *Brucella*-positive samples were found, five brine cheeses, one cream and one hard cheese made of ovine or mixed milk. In Greece, a brucellosis control and eradication program for sheep and goats has been implemented but diverging policies and measures in different regions hamper its success. On the Greek islands, where ovine and caprine brucellosis is still endemic, a test-and-slaughter policy is in place whereas on the Greek mainland (as well as on some of the islands, including Lesvos and Leros), where the prevalence of brucellosis is higher, a control strategy is carried out by official mass vaccination of young and adult sheep and goats using the Rev-1 vaccine strain. In 2011, 912,790 sheep and goats from 23,080 flocks were vaccinated (EFSA and ECDC, 2014). Similar sanitary measures are taken in Spain, which is also not OBF/ObmF. Unsurprisingly, we detected a pasteurized *Brucella*-positive Spanish hard cheese made of mixed milk. We assume that either milk from infected or vaccinated animals was used for cheese production. Though both real-time PCR assays applied in our study may not differentiate the field from vaccine strains, in any case, the epidemiological impact can be high. Live-attenuated vaccines are not commonly shed in milk but recently raised nationwide awareness in Texas and New Jersey (United States of America) where human cases infected by *B. abortus* vaccine strain RB51 after raw milk consumption were reported (CDC, 2017; Cossaboom et al., 2018).

Taking a closer look at the cheese samples imported from non-EU MS, we detected *Brucella* DNA in pasteurized cheese mainly from Turkey. In total, 43 countries including Turkey are listed as approved to introduce pasteurized, ultra-high temperature treated or sterilized milk and dairy products (EC–European Commission, 2018). All 12 Turkish cheese samples containing *Brucella* DNA (3 cream cheeses, 3 soft cheeses and 6 semi hard cheeses) were pasteurized. Prevalence data from Turkey show that up to 14.3% of the investigated cheese samples may be contaminated by *Brucella* spp. (Kasimoğlu, 2002). Moreover, human *Brucella* isolates (predominantly *B. melitensis*) from German travellers and Turkish immigrants living in Germany revealed epidemiological concordance with sheep isolates originating from Eastern Anatolia, Turkey (Gwida et al., 2010). Bulgaria which was by the time of the study non-EU MS had no trade agreement with the EU and was not included in the positive list for the import of raw milk cheese into the EU. Consequently, the 10 Bulgarian brine cheeses, a cream cheese and a semi hard cheese might have been imported illegally. In contrast, the non-EU MS Lebanon signed an Association Agreement with the EU in June 2002, which entered into force in April 2006. As a result, Lebanese industrial as well as most agricultural products benefit from free access to the EU market.

4.4. Spatio-temporal distribution of Brucella in cheese

We detected *Brucella* DNA significantly more often in late than in early summer purchases. The brucellosis incidence in endemic countries is known to peak in early summer, four weeks after lamb slaughter, when the production and consumption of fresh cheese begins (De Massis, Di Girolamo, Petrini, Pizzigallo, & Giovannini, 2005). The early summer peak in endemic countries is mainly due to occupational exposure during lambing and slaughtering whereas cases in non-endemic European countries, such as Germany, are typically reported in the late summer months after the migrant population returns from holidays spent in endemic regions of their home country in the Mediterranean area (Al Dahouk et al., 2007b). A similar seasonal peak of human infections in summer was also described for other foodborne zoonoses such as campylobacteriosis, salmonellosis and VTEC (EFSA and ECDC, 2017) which might have been caused by enhanced pathogen survival rates and proliferation triggered through higher temperatures (Lal, Hales, French, & Baker, 2012). The sampling in our study was deliberately planned for early and late summer months because we expected a higher *Brucella* load in cheese. Since the cheese was purchased over a limited period of time and only in a single year, a sampling bias has to be assumed.

Interestingly, clusters of more than 50% positive samples emerged in the cheese sold by nine vendors. Our purchase from one specific market vendor revealed even 100% positive samples, which were bought at the same date but were highly heterogeneous in terms of geographical origin, cheese and milk type. Similarly, the purchases from two other market vendors showed a high rate of positive samples, with 80% and 75%. Cross contamination during laboratory investigations can be ruled out owing to the setup of the analyses separating inner core and the cheese rind. We suppose that these clusters of positive cheeses can be explained by close trade links of the vendors to dairy farmers and production sites in endemic countries in combination with large-scale illegal import and a prosperous black market. The illegal import of foodstuff from brucellosis endemic countries into Germany has been recently described in an airport study: A total of 663 food items were seized from 296 air passengers arriving in Germany from 35 different departure countries between 2012 and 2013 (Beutlich et al., 2015). The majority of confiscates (51%) originated from Turkey and Russia. A selection of 474 samples was subjected to microbiological analyses. Seventeen food products were *Brucella*-positive (using the same genus-specific real-time PCRs targeting *bcsp31* and IS711 as we did). About half of the *Brucella*-positive food items were illegally imported from Turkey (53%, n = 9). Eight products were cheeses, four of which were homemade. From one of the homemade cheeses verotoxin-producing *E. coli* was also isolated. *Brucella* was further detected in a raw meat product from Turkey. Other countries of origin of illegal *Brucella*-positive food imports were Russia, South Africa, China and South Korea. In total, 10 *Brucella*-positive food items were commercial products, 6 homemade and one raw foodstuff. *Brucella* could not be isolated from the PCR-positive food samples which might be due to the low numbers of pathogenic bacteria in the food matrix, the competing food microbiome or a viable but non-culturable (VBNC) status (Beutlich et al., 2015). These results were underpinned by a Brazilian study, which detected *Brucella* in 42% (70/166) of illegally imported dairy products, originating from Argentina, France, Iraq, Israel, Italy, Lebanon, Portugal, Spain and Turkey. *Brucella* was mainly detected in cheese (n = 62) from endemic regions, including Portugal (n = 10) and Italy (n = 23) (Barros de Melo et al., 2014). Our findings suggest that certain vendors run their business by illegal imports. Whereas smaller quantities of illegally imported food by individuals may be rather

considered for personal use, larger quantities might be ordered and distributed via retailers or sold on (black) street markets as deliberate act, predominantly based on commercial grounds (Chaber, Allebone-Webb, Lignereux, Cunningham, & Rowcliffe, 2010; Nagy et al., 2015). Desired consumer satisfaction may be the driving force behind the fraudulent information on raw milk content.

5. Summary and conclusions

Despite its widespread distribution and global impact, brucellosis became an underestimated zoonosis in the EU where travel- and import-associated cases prevail (Corbel, 2006). In Europe, travels to endemic regions surrounding the Mediterranean Sea such as Turkey and the Middle East lead to a higher risk of infection (Al Dahouk et al., 2007b; Gwida et al., 2012). However, about a quarter of the brucellosis patients in Germany do not have an appropriate travel history. In these cases, illegally imported dairy products contaminated with *Brucella* spp. may play a decisive role (Beutlich et al., 2015). Hence, the free movement of goods on the inner European market and increasing imports from third countries challenge day by day the high level of food safety applied by the European Union. In our epidemiological study, 200 cheese samples from weekly markets, ethnic delis and supermarkets were investigated for the presence of *Brucella*. As most brucellosis patients in Germany have a migration background, the samples were taken in districts of Berlin with a large migrant community. In total, the prevalence of *Brucella* DNA positive cheese was 20.5%. Clustered positive samples at certain vendors suggest organized trade of illegal imports from endemic regions. A few cheeses investigated were produced from raw milk and had a short ripening period. Therefore, the survival of *Brucella* in the cheese matrix seems to be possible, although no viable bacteria could have been isolated. Nonetheless, our findings may help to explain autochthonous *Brucella* infections in Germany affecting patients without travel history to endemic countries. In summary, even in non-endemic countries consumers should be educated about potential health risks related to the consumption of raw animal products imported from endemic regions. However, consumers cannot easily identify hazardous dairy products since most cheeses are sold with fraudulent intent and misleading information is provided. Official sanitary control measures should therefore focus on pathogen detection and product quality in all segments of the food market.

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